Biofilm system 2

FISH, FACS and Cell Counts

Introduction

Fluorescent *in situ* hybridization (FISH) is a method that today is widely used for identification, visualization and localization of micro-organisms in many fields of microbiology.

In 1989, DeLong *et al* reported the first *in situ* hybridization using fluorescently labelled ribosomal RNA-directed oligonucleotide probes

Fluorescence *in situ* hybridization is normally based on short oligonucleotides which are complementary to an rRNA signature sequence and have a fluorescent dye molecule attached to the 3' or the 5'-end. Polynucleotide probes and labelling with several fluorochrome molecules have also been used. The probes target a specific complementary sequence on the rRNA in the intact cells. In most cases, one can design probes that hybridize with rRNA from only a single species or even subspecies, as well as probes that target a broader taxonomic unit (e.g. genus or phylum). In combination with fluorescent microscopy or confocal laser scanning microscopy (CLSM) it is possible not only to identify the organisms but also to reveal their precise location in a three-dimensional community. FISH has been used extensively in environmental microbiology, enabling investigators to map complex structured microbial communities, but today FISH is also used in clinical microbiology and for diagnostic purposes.

General procedure for FISH

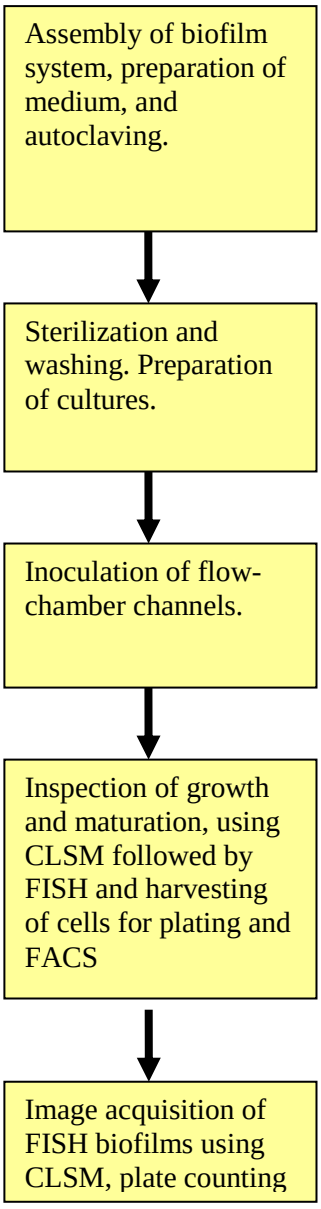
FISH is a rapid method for visualization and identification of bacteria directly in samples of interest and the technique is able to reveal non-culturable species. However, if phylogenic identification is not required, general DNA stains, like the SYTO stains (Molecular Probes) which have been widely developed during the last years, may be very useful. In short the FISH procedure is as follows: The sample is fixed using an aqueous solution of paraformaldehyde for Gram-negative bacteria, and 50% ethanol or heat treatment for Gram-positive bacteria. In some cases it is important that three-dimensional properties in a sample are retained, and for that purpose samples can be embedded in an acryl amide or agarose gel after fixation. After fixation and possibly embedding the bacterial sample is hybridized with a fluorescently labelled probe that is complementary to the 16S rRNA in the cells. Stringency of the hybridization has to be optimized for the specific probe sequences used. After incubation unspecific probe has to be removed by several washing steps before the sample can be mounted for visualization using fluorescent or confocal microscopy.

Aims

In the present exercise you are going to establish a mixture of *P. aeruginosa* (Gfp-tagged) with two variants of *Acinetobacter* (untagged). You will label the *P. aeruginosa* with a blue fluorescent CY5 probe, and the used *Acinetobacter* strain with a red fluorescent CY3 probe: When a mature biofilm population has developed you will perform FISH for visualization of the spatial distribution and organization of the different strains in the community. Furthermore, you are going to harvest channels of the community followed by plating on selective plates and FACS sorting for estimation of the ratio between the strains after biofilm growth.

Reference:

DeLong et al. Phylogenetic Stains: Ribosomal RNA-Based Probes for the Identification of Single Cells. *Science* 1989 243:1360-1363


Assembly of biofilm system, preparation of medium, and autoclaving.
Sterilization and washing. Preparation of cultures.
Inoculation of flow-chamber channels.
Inspection of growth and maturation, using CLSM followed by FISH and harvesting of cells for plating and FACS
Image acquisition of FISH biofilms using CLSM, plate counting

Experimental procedures

Day 1. Assembly of the biofilm system, preparation of medium, and autoclaving.

See the protocol for biofilm work.

Day 2. Sterilization and washing of the biofilm system, equilibrate tubing with media over night.

See the protocol for biofilm work.

Day 2. Preparation of cultures.

Each team will have one flow cell available (3 channels) and a mixture of the following strains should be inoculated in each channel (one channel is used for FISH and two for harvesting followed by plating and FACS sorting).

- ***P. aeruginosa*** **Gfp tagged diluted 1000 times in 0.9% NaCl,**
- ***Acinetobacter*** **variants diluted 10 times in 0.9% NaCl**

The strains should be inoculated in 10 mL LB and incubated O.N. at 30°C

Day 3 (morning). Inoculation of flow-chamber channels.

See the protocol for biofilm work

The O.N. cultures should be diluted and mixed as specified above before inoculation.

Day 6. Inspection of biofilms followed by FISH and harvesting of biofilm populations.

After inspection of mature biofilms all tree channels are fixed using freshly prepared 4% paraformaldehyde (see biofilm protocol). After one hour incubation with fixative the cells are washed with PBS and then two of the tree channels are harvested using small glass beads that are introduced in the channels to remove the biofilm population from the flow chambers by shear force. The harvested cells are collected in eppendorf tubes and homogenized by continuously pumping it in and out a small syringe with a 0.5 gauge needle for a couple of minutes.

After this the cells can be FACS sorted and plated for determination of CFU/ml on selective plates. Gentamycin plates (20 µg/ml) for *P. aeruginosa*, streptomycin plates (100 µg/ml) for *Acinetobacter*. LB plates without antibiotics are used for total count and control for contamination.

FISH is performed on the last channel and as specified in the FISH protocol for biofilms.

Day 7. Finishing up the FISH and image acquisition

All microscopic observations are performed by the use of a Zeiss LSM510 Confocal Laser Scanning Microscope (CLSM) equipped with lasers, filter sets, and detectors for monitoring of Gfp (excitation 488 nm, emission 517nm) and red-fluorescence emitted from the CY3 probe (excitation 534 nm, emission 565 nm); as well as blue-fluorescence emitted from the CY5 probe (excitation 633 nm, emission 650 nm). The images are acquired with a 40x/1.3 Plan-Neofluar oil immersion objective.

Inspect plates and count colonies.

Image analysis.

Prepare images of the different biofilms using Imaris software.